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(71) Applicant: CHIRON CORPORATION  
4560 Horton Street  
Emeryville California 94608(US)

(72) Inventor: Burke, Rae Lyn  
1624 8th Avenue  
San Francisco California(US)

(72) Inventor: Tekamp/Olson, Patricia  
1426 43rd Avenue  
San Francisco California(US)

(72) Inventor: Rosenberg, Steven  
3861 Fruitvale Avenue  
Oakland California(US)

(72) Inventor: Valenzuela, Pablo D. T.  
455 Upper Terrace No. 3  
San Francisco California(US)

(74) Representative: Glawe, Delfs, Moll & Partner  
Patentanwälte  
Postfach 26 01 62 Liebherrstrasse 20  
D-8000 München 26(DE)

(54) Yeast expression systems with vectors having GAPDH or PyK promoters, and synthesis of foreign proteins.

(57) Yeast cells containing DNA plasmids having foreign DNA, wherein foreign DNA is expressed, are described. For example, DNA coding for hepatitis B and its virus surface antigen (HBsAg) is ligated to a yeast plasmid to yield a product that is used to transform yeast cells. The plasmids of this invention have either GAPDH or PyK promoters, and are capable of replicating in either a yeast cell or a bacterial cell.

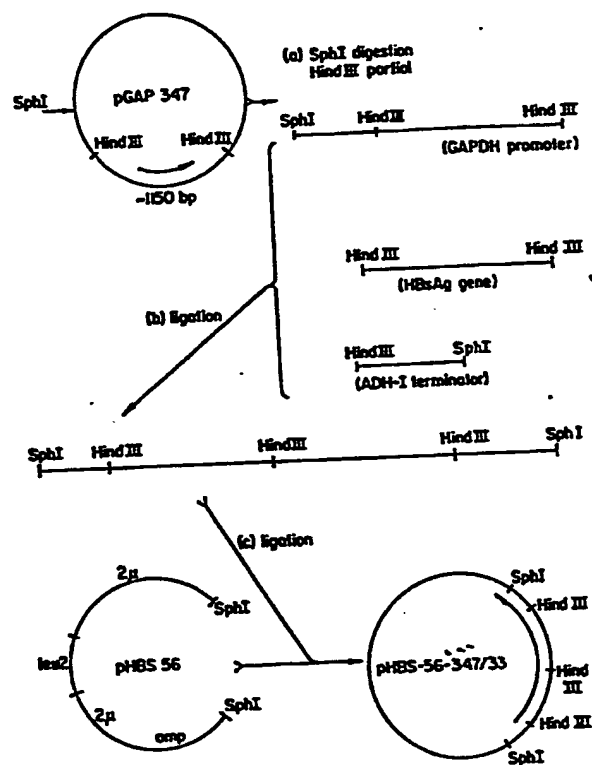


FIG. 3.

9729-12

YEAST EXPRESSION SYSTEMS WITH VECTORS HAVING GAPDH  
OR PyK PROMOTERS, AND SYNTHESIS OF FOREIGN PROTEIN

5

BACKGROUND OF THE INVENTION

For maximal expression of foreign genes in microbial systems it is usually advantageous to employ homologous regulatory elements within the expression vector. Efficiency of expression (product formation) is believed to be a function of and proportional to the strength of the promoter employed. In addition, regulation of gene expression by nutritional factors under the control of the experimenter offers a further useful manipulatory tool. The glycolytic enzyme genes of yeast, e.g., those coding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate kinase (PyK), possess the above useful properties, i.e., high levels of expression (and thus by inference very efficient promoters) and susceptibility to regulation by components of the growth medium. For example, GAPDH can comprise as much as 5% of the dry weight of commercial baker's yeast (Krebs, E.G., J. Biol. Chem. (1953) 200:471). Furthermore, these enzymes are also highly inducible. For example, when yeast cultures are shifted from growth on acetate to glucose, the activity of GAPDH increased up to 200-fold in proportion to the concentration of the sugar in the medium (Maitra, P.K. and Lobo, Z., J. Biol. Chem. (1971) 246:475). These results suggest that the transcriptional machinery of these genes is highly regulated, perhaps by the participation of DNA sequences present in the 5' non-coding flanking region of the genes.

This invention relates to the isolation, structure and the successful use in yeast expression plasmids of DNA fragments corresponding to the 5' non-coding regions of the regulatable yeast genes GAPDH and PyK. These fragments which contain DNA sequences

with strong transcription-promoting activity are called "promoters". They are ideal components of DNA vectors for commercial production of large quantities of protein coded by foreign genes under their transcriptional control.

5 In addition, this invention encompasses yeast expression plasmids further comprising an appropriate terminator to form a "cassette" of promoter-foreign gene-terminator. The presence of the terminator  
10 increases expression of the foreign DNA.

An early attempt to express foreign DNA in yeast failed (Beggs, J.D. et al., Nature (1980) 283:285). In this report, the hemoglobin DNA (inserted with its own promoter) was transcribed but the RNA was  
15 not spliced. A variety of explanations for this result are possible, e.g., an incorrect location for the initiation of transcription and/or the poor ability of yeast cells to carry out splicing of intervening sequences (introns).

20 Three GAPDH genes of yeast have been cloned (Holland, M.J. et al., Basic Life Science (1981) 19:291), but their promoters have not been used for constructing expression systems in yeast by recombinant DNA methods. The PyK gene has also been cloned, but by  
25 genetic complementation only (no structural studies performed) (Kawasaki, G. and Fraenel, D.G., Biochem. Biophys. Res. Comm. (1982) 108:1107). Other yeast promoters, e.g., that of alcohol dehydrogenase I (Valenzuela, P. et al., Nature (1982) 298:347 and  
30 Hitzeman, R.A. et al., Nature (1981) 293:717) and phosphoglycerate kinase (Tuite, M.F. et al., EMBO J. (1982) 1:603 and Hitzeman, R.A. et al., Science (1983) 219:620) have been linked to foreign genes to produce yeast expression but no terminators were used. The  
35 present invention provides new promoters for yeast expression systems and combines the advantages of

highly expressive promoters with the enhanced expression found with appropriately ligated terminators.

#### BRIEF DESCRIPTION OF THE INVENTION

This invention relates to a yeast expression  
5 vector comprising a segment of foreign DNA, e.g., that  
coding for hepatitis B virus (HBV) surface antigen  
(HBsAg), under transcriptional control of either a  
yeast GAPDH promoter or a yeast PyK promoter. Termina-  
tors may also be appropriately attached. The expression  
10 vector typically has a yeast replication origin and a  
bacterial replication origin and is capable of repli-  
cating in either type of cell. The expression vector,  
when used to transform yeast cells, will yield  
substantial amounts of the protein coded by the segment  
15 of foreign DNA.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: Isolation and tailoring of a GAPDH  
promoter fragment.

20 Fig. 2: DNA sequence of the GAPDH promoter  
fragment.

Fig. 3: Construction of a yeast expression  
plasmid containing the GAPDH promoter.

Fig. 4: Nucleotide sequence of the pyruvate  
kinase (PyK) gene.

25 Fig. 5: Construction of a yeast expression  
plasmid containing the PyK promoter region.

#### DETAILED DESCRIPTION OF THE INVENTION

In principle, yeast expression plasmids have  
particular advantages, including the following. Yeast  
30 can be grown in large-scale culture for commercial  
production by processes well-known in the art. In  
contrast, bacteria in large-scale culture are subject  
to the frequent problem of "phage-out". Yeast also  
appears to have much the same ability as mammalian  
35 cells to add carbohydrate groups to newly synthesized  
proteins, a capacity that bacteria do not have. Now

that cDNA sequences are readily obtainable, the problem of expressing genes having introns is easily avoided.

The vectors of the present invention encompass promoters of unusually high efficiency. A promoter is defined herein as a DNA segment capable of functioning to initiate transcription of an adjoining DNA segment. Transcription is the synthesis of RNA (herein termed messenger RNA or mRNA), complementary to one strand of the DNA adjoining the promoter region. In eukaryotes, messenger RNA synthesis is catalyzed by an enzyme termed RNA polymerase II. The minimum essential elements of promoter function are the following: To provide a starting point for the initiation of transcription and to provide a binding site for RNA polymerase II near the start site permitting selection of the proper strand of DNA as a template for messenger RNA synthesis. In addition, a eukaryotic promoter functions to regulate the relative efficiency of transcription of coding segments under its control. An active promoter is one which elicits synthesis of relatively large amounts of mRNA complementary to a strand of the adjacent DNA coding segment.

The structural correlates of promoter function have not been clearly established. A promoter segment usually can be identified in nature as a region lying adjacent to the 5'-end of a given structural gene. (References to the 5' and 3' ends of a gene will be understood to indicate the corresponding respective ends of mRNA transcribed therefrom, and these, in turn, will be understood to correlate with the NH<sub>2</sub>- and -COOH termini of the encoded protein, respectively.) Comparisons of the nucleotide sequences of promoters for various genes from various species have revealed only a few short regions of nucleotide sequence similarity in common among them. Most notable of these is the "TATA Box," a segment of about 5 to 10 nucleotides located generally about 70 to 230 nucleotides upstream

from the site of transcription initiation, having a sequence generally resembling TATAA. For review of structural comparisons see Breathnach, R. and Chambon, P., Ann. Rev. of Biochem. (1981) 50:349. The TATA Box  
5 is believed to function in initiation of transcription.

The foreign gene will be free or substantially free of codons from the normal structural gene associated with the promoter. Usually, the foreign gene will be joined to a non-coding 3'-end of the regulatory  
10 region encompassing the promoter, so as to be free of the amino acids at the N-terminus of endogenous gene naturally associated with the regulatory region. That is, fewer than about 3 codons (9 nucleotides) will be retained with the regulatory region when joined to the  
15 foreign gene.

The presence of the terminator sequence at the 3' end of the coding segment enhances expression. The effect is generally similar to the addition of rho factor to prokaryotic transcription systems, wherein  
20 the rate of the release of RNA polymerase is enhanced to produce an increase in the rate of reinitiation of transcription. It will be understood that, while the terminator sequences are not required for detectable expression of foreign DNA segments, it is preferable to  
25 appropriately link them to enhance expression. The terminator region may be naturally associated with the same or different structural gene as the promoter region.

The most appropriate DNA vector for the GAPDH  
30 or PyK construction of this invention is a shuttle vector. These vectors can "shuttle" between a bacterial strain, such as E. coli, and yeast, since they have a bacterial origin of replication and a yeast origin of replication, see, e.g., Ammerer, G. et al., Recombinant  
35 DNA, Proc. Third Cleveland Symposium Macromolecules (Walton, A.G., ed.), p. 185, Elsevier, Amsterdam (1981). A typical bacterial origin of replication is

derived from, e.g., pBR322. The most useful yeast origin of replication is found in the extrachromosomal genetic element known as the 2 micron circle. In laboratory strains the 2 micron plasmid DNA is found in  
5 approximately 50 copies per cell and is stably maintained. For a review, see, for example, Curr. Topics Micro. Imm. (1982) 96:119. This yeast plasmid has also been sequenced (Hartley, J.L. et al., Nature (1980) 286:860).

10 Representative samples of the plasmids and host cells used in the constructions of this invention have been placed on deposit with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland. Plasmid pPyK 9.1.1 and yeast cell trans-  
15 formants 2150-2-3/pHBS-56 GAP347/33 and 2150-2-3/pHBS56PyK were placed on deposit on February 18, 1983 and have received ATCC Accession numbers 40061, 20665 and 20666, respectively.

In the Examples that follow, many of the  
20 techniques, reactions and separation procedures are already well-known in the art. All enzymes, unless otherwise stated, are available from one or more commercial sources, such as New England Biolabs, Beverly, Massachusetts; Collaborative Research, Waltham,  
25 Massachusetts; Miles Laboratories, Elkhart, Indiana; Boehringer Biochemicals, Inc., Indianapolis, Indiana and Bethesda Research Laboratories, Rockville, Maryland. Buffers and reaction conditions for restriction enzyme digestion were used according to recommendations  
30 supplied by the manufacturer for each enzyme, unless otherwise indicated. Standard methodology for other enzyme reactions, gel electrophoresis separations and E. coli transformation may be found in Methods in Enzymology, (1979) 68. Transformation of yeast proto-  
35 plasts can be carried out essentially as described by Beggs, Nature (1978) 275:104.



E. coli strains useful for transformation include X1776; K12 strain 294 (ATCC No. 31446); RR1 and HB101. Yeast strains XV610-8c having the genotype (ade2 ade6 leu2 lys1 trp1 can1) and GM-3C-2, genotype: (Leu2 Trp1 His4 CYC1-1CYP3-1) (Faye, G. et al., Proc. Natl. Acad. Sci. (1981) 78:2258) can be typically used for yeast transformations. It would be understood, however, that virtually any strain of yeast is useful for transformation. Bacteria can be grown and selected according to procedures described by Miller, J.H., Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1972). Yeast can be grown on the following media: YEPD containing 1% (w/v) yeast extract, 2% (w/v) peptone and (w/v) glucose; and, in the case of plating medium, 3% (w/v) agar. YNB plus CAA contains 6.7 grams of yeast nitrogen base (Difco Laboratories, Minneapolis, Minnesota), 10mg of adenine, 10mg of uracil, 5g casamino acids (CAA) (Difco), 20g glucose; and, in the case of plating media, 30g agar per liter. Selection for tryptophan prototrophy can be made on plates containing 6.7g yeast nitrogen base (lacking amino acids), supplemented for all growth requirements of the strain to be transformed except tryptophan.

25

#### EXAMPLE 1

##### Cloning of the yeast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

A complementary DNA (cDNA) containing the yeast GAPDH coding sequences was prepared in the following manner:

PolyA+ RNA was isolated from yeast strain A364A. Double-stranded cDNA was synthesized using AMV reverse transcriptase and E. coli DNA polymerase I. Poly-dC-tails were added to the double-stranded cDNA molecule using deoxynucleotide terminal transferase. Poly-dC-tailed cDNA was annealed to poly-dG-tailed

pBR322 and used to transform E. coli HB101. One thousand transformants were screened by colony hybridization to labeled PolyA+ RNA, and a subset further examined by restriction endonuclease mapping, and DNA sequencing. Three clones containing GAPDH sequences were isolated from the pool. One clone (pcGAP-9) contained an insert of about 1200 base pairs (bp) and was used for further work.

A yeast gene library was prepared by inserting fragments obtained after partial digestion of total yeast DNA with restriction endonuclease Sau3A into lambda phage Charon 28, according to Blattner, F.R. et al., Science (1977) 196:161-169. Several fragments containing yeast GAPDH coding sequences were isolated by screening the phage library with labeled DNA from pcGAP-9. The yeast GAPDH gene of one of these clones was subcloned in pBR322 as a 2.1kb HindIII fragment (pGAP-1, see Fig. 1) or as a 3.5kb BamHI fragment (pGAP-2). The GAPDH promoting-active fragments were isolated from these clones. The HindIII-HhaI fragment of about 800bp was ligated to the HhaI-HindIII fragment of about 350bp. The resulting 1061bp HindIII fragment was isolated by gel electrophoresis and cloned in pBR322, (pGAP-347), and the sequence determined (see Fig. 2).

## EXAMPLE 2

### Construction of yeast vectors containing the GAPDH promoter, active in the expression of HBsAg.

A plasmid vector (pHBS-56GAP347/33), for the expression of HBV surface antigen in yeast, using the GAPDH promoter fragment was constructed as depicted in Fig. 3.

Total digestion of pGAP-347 with SphI followed by partial digestion with HindIII yielded an approximately 1700bp SphI-HindIII fragment having about 1060bp of GAPDH promoter and about 530bp of pBR322. The

1700bp SphI-HindIII GAPDH promoter fragment was ligated with the 840bp HindIII-HindIII fragment (containing the HBsAg coding region, 26 bases of 5' non-coding region and 128bp of 3' non-coding region, obtained from pHS-56) and then with the 350bp HindIII-SphI fragment containing the ADH-1 termination region (isolated from pHS-56). The 2900bp SphI fragment (cassette) was isolated and cloned in pHS-56 previously digested with SphI. The plasmid pHS-56 (ATCC Accession No. 40047) has been described in a co-pending application (EPA No. 82.401473.2 published as no. 72318, of Regents of the University of California, herein incorporated by reference) and contains the entire 2 micron plasmid, in addition to a region with the yeast leu2 gene and the amp resistance locus of pBR322. The resulting plasmid (pHS-56GAP347/33) in which the promoter, gene and termination regions were in the proper orientations was isolated and used to transform yeast strain AB102 (MATa, pep 4-3, leu 2-3 leu2-112, ura 3-52, his 4-580, cir<sup>o</sup>) or strain 2150-2-3 (MATa, adel, leu2-04, cir<sup>o</sup>). Strain AB102 is derived from SF657-9c by curing of 2 micron plasmids. Strain 2150-2-3 is from the collection of Dr. Leland Hartwell at the University of Washington.

25

EXAMPLE 3

Synthesis of HBsAg in yeast under GAPDH promoter control (plasmid pHS-56GAP347/33).

One hundred ml cultures of strain AB102 containing plasmid pHS56-347/33 were grown to optical density at 650nm of 1. Cell-free lysates were prepared by agitation with glass beads and removal of cell debris by centrifugation. HBsAg was measured by the Abbott AusriaII radioimmunoassay and protein concentration was determined by the Coomassie blue binding method. The results are shown in Table 1. They indicate that the GAPDH promoter is about 5 times more

effective than the ADH-1 promoter for protein product expression in yeast.

Table 1: Synthesis of HBsAg in yeast

(a) control from pHBS-56 (ADH-I promoter)				
5	<u>Exp#</u>	<u>sAg</u> ( $\mu\text{g/ml}$ )	<u>protein</u> ( $\text{mg/ml}$ )	<u>Spec. Activity</u> ( $\mu\text{gsAg/mg protein}$ )
	1	8.8	18	0.49
	2	14	25	0.56
	3	12.4	20	0.62
(b) from pHBS-56GAP347/33 (GAPDH promoter)				
10	<u>Exp#</u>	<u>sAg</u> ( $\mu\text{g/ml}$ )	<u>protein</u> ( $\text{mg/ml}$ )	<u>Spec. Activity</u> ( $\mu\text{gsAg/mg protein}$ )
	1	36	14	2.6
	2	35	12	2.9
15	3	37	12.5	3.0

Similar results were obtained by substituting yeast strain 2150-2-3 for yeast strain AB102 and repeating Example 3.

#### EXAMPLE 4

##### 20 Cloning of the yeast pyruvate kinase gene.

The pyruvate kinase gene was cloned by complementation. A yeast pyruvate kinase minus mutant was transformed with a pool of recombinant YEp24 plasmids containing wild type yeast genomic DNA. The yeast strains S288C (genotype: SUC2, mal, gal2, CUP1) and pyk 1-5 (genotype: a, adel, leu1, met14, ura3, pyk1-5) were obtained from the Yeast Genetic Stock Center, Department of Biophysics, University of California, Berkeley. The yeast genomic bank used consists of a partial Sau3A digest of total DNA from

the strain S288C cloned into the BamHI site of the "shuttle" vector YEp24. The vector YEp24 contains pBR322 sequences for selection and growth in bacteria, the yeast URA3 gene for selection in yeast and an EcoRI  
5 fragment of the yeast 2 $\mu$  circle to ensure plasmid replication and segregation in yeast. The pool includes sufficient independent recombinant plasmids to represent the entire yeast genome.

The strain pyk1-5 is unable to grow on medium  
10 containing glucose or lacking uracil because of mutations in this strain at the Pyk1 and URA3 loci, respectively. Transformation of this strain with the YEp24 genomic library and selection for transformants which are able to grow on medium lacking uracil and  
15 containing glucose selects for those cells which have acquired YEp24 containing the pyruvate kinase gene. Transformation of  $3.5 \times 10^8$  pyk1-5 yeast cells with 10 $\mu$ g of YEp24 recombinant plasmid pool DNA yielded 5 independent transformants which grew in the absence of  
20 uracil and the presence of glucose.

Characterization of the insert DNA of these transformants by restriction enzyme analysis indicated that they contained overlapping DNA inserts. We focused on a single transformant, pPyK 9.1, which  
25 contained a 7.0kb insert. The pyruvate kinase gene was localized within this insert by determining which insert-specific restriction fragments hybridized to a mRNA of about 1.7kb expected for the pyruvate kinase mRNA. The localization of the PyK gene was confirmed  
30 by subcloning appropriate regions of the insert DNA and observing complementation of function in the pyk1-5 mutant. A subclone pPyK 9.1.1 which contained the PyK gene on a 4.4kb insert was sequenced and used in expression plasmid constructions.

EXAMPLE 5Sequence of the yeast pyruvate kinase gene.

A total of 2885 nucleotides of the PyK gene have been sequenced including 1497 nucleotides in a single open reading frame, 911 nucleotides of 5' untranslated region and 477 nucleotides of 3' untranslated region (see Fig. 4). The gene encodes a polypeptide of 499 amino acids to give a monomer molecular weight of 54,608 daltons which agrees well with the expected value for yeast PyK. The amino acid composition derived from the nucleotide sequence also corresponds closely with that measured from the isolated yeast protein. The nucleotide sequence predicts a carboxy terminal valine which has been found for yeast pyruvate kinase.

EXAMPLE 6Construction of yeast expression plasmids using the pyruvate kinase promoter region.

Two different constructions were made: pHBS16 PyK and pHBS56 PyK. The procedures are outlined in Fig. 5.

The plasmid pPyK 9.1.1, which contains the yeast PyK gene cloned in pBR322 was digested with XbaI and the protruding ends filled in with deoxynucleotides using DNA polymerase I. The product was digested with BamHI to finally isolate a 912bp BamHI-blunt fragment containing the PyK promoter and 8 bases from the PyK coding region. This fragment was ligated to plasmid pHBS-6 (contains the HBSAg gene, in which the 5' non-coding region has been deleted, cloned in pBR322) previously digested with NcoI, filled in using DNA polymerase and digested with BamHI. After transformation of E. coli, pHBS-6PyK was isolated. This plasmid contains the PyK promoter with codons for 3 extra amino acids fused in phase with the HBSAg coding sequence,

ATGTCTAG, CATG, .

pHBS-6PyK was digested with BamHI to completion and partially digested with EcoRI to isolate a 1750bp BamHI-EcoRI fragment containing the PyK promoter fused to the HBsAg gene. This 1750bp fragment was ligated to  
5 the large fragment obtained after digestion of pHBS-16 (ATCC Accession No. 40043, plasmid described in European Patent Application No. 82.401473.2 mentioned above)

with BamHI

10 and EcoRI and used to transform E. coli. The yeast expression plasmid pHBS-16PyK was obtained. pHBS-16PyK was digested to completion with SphI and XbaI and a 1200bp SphI-XbaI fragment (containing 200bp of pBR322, the PyK promoter and 100bp of the 5' region of the  
15 HBsAg gene) was isolated. This 1200bp SphI-XbaI fragment was ligated to a 1070bp XbaI-SphI fragment (isolated from pHBS-56) containing the 3' end of the HBsAg gene and the ADH-1 terminator. After digestion with SphI, a SphI-SphI 2300bp fragment (cassette)  
20 containing the PyK promoter, HBsAg gene and ADH-1 terminator was isolated. This cassette fragment was cloned in pHBS-56 which had been previously digested with SphI. The yeast expression plasmid pHBS-56 PyK was obtained. This plasmid was used to transform yeast  
25 strain AB102 (see Example 2) or strain 2150-2-3 (see Example 2).

#### EXAMPLE 7

##### Synthesis of HBsAg in yeast under PyK promoter control.

30 One hundred ml cultures of strain AB102 containing plasmid pHBS-56 PyK were grown to optical densities at 650nm of 1-2. Cell-free lysates were prepared by agitation with glass beads and removal of cell debris by centrifugation. HBsAg was measured by  
35 the Abbott AusriaII radioimmunoassay and protein concentration was determined by the Coomassie blue binding method. The results are shown in Table 2.

They indicate that PyK promoter is at least two times more efficient than the ADH1 promoter for expression of protein product in yeast.

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Table 2: Synthesis of HBsAg in yeast

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5 (a) from pHBS-56 (control, ADH-I promoter)

<u>Exp#</u>	<u>sAg</u> ( $\mu\text{g/ml}$ )	<u>protein</u> ( $\text{mg/ml}$ )	<u>Spec. Activity</u> ( $\mu\text{gsAg/mg protein}$ )
1	8.2	24	0.34
2	7.2	24	0.32
10 3	4.7	27	0.23

(b) from pHBS-56 PyK (PyK promoter)

<u>Exp#</u>	<u>sAg</u> ( $\mu\text{g/ml}$ )	<u>protein</u> ( $\text{mg/ml}$ )	<u>Spec. Activity</u> ( $\mu\text{gsAg/mg protein}$ )
1	18	2.5	0.68
15 2	10.6	22	0.48
3	15.2	27	0.56

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Similar results were obtained by substituting yeast strain 2150-2-3 for yeast strain AB102 and repeating Example 7.



CLAIMS

1. A yeast expression vector comprising a segment of foreign DNA under transcriptional control of a yeast glyceraldehyde-3-phosphate dehydrogenase promoter, said segment being in the correct orientation for transcription and substantially free of codons from yeast glyceraldehyde-3-phosphate dehydrogenase at the 5'-end of said foreign DNA.
2. A yeast expression vector comprising a segment of foreign DNA under transcriptional control of a yeast pyruvate kinase promoter, said segment being in the correct orientation for transcription and substantially free of codons from yeast pyruvate kinase at the 5'-end of said foreign DNA.
3. A yeast expression vector of claim 1 or claim 2 further comprising a terminator attached to the 3' end of the segment of foreign DNA.
4. A yeast expression vector according to any of claims 1 and 2, further comprising yeast two micron plasmid DNA or portion thereof.
5. A yeast expression vector of any claims 1 and 2 wherein said foreign DNA codes for hepatitis B surface antigen or portion thereof.
6. The plasmid pHBS-56GAP347/33.
7. The plasmid pHBS-56 PyK.
8. A method of expressing a DNA coding segment in yeast, comprising the steps of:
  - (a) inserting the coding segment in a yeast expression vector, said vector comprising a DNA segment derived from a yeast glyceraldehyde-3-phosphate dehydrogenase promoter substantially free of codons from the 5'-end of yeast glyceraldehyde-3-phosphate dehydrogenase, said promoter being adjacent to the 5' end of the inserted DNA coding segment and so oriented that transcription initiated within said promoter includes the coding segment, thereby providing a coding segment expression vector, and

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(b) transforming yeast cells with the coding segment expression vector.

9. A method of expressing a DNA coding segment in yeast, comprising the steps of:

- 5 (a) inserting the coding segment in a yeast expression vector, said vector comprising a DNA segment derived from a yeast pyruvate kinase promoter substantially free of codons from the 5'-end of the yeast pyruvate kinase, said promoter being adjacent  
10 to the 5'-end of the inserted DNA coding segment and so oriented that transcription initiated within said promoter includes the coding segment, thereby providing a coding segment expression vector, and

(b) transforming yeast cells with the coding  
15 segment expression vector.

10. A method according to any one of claims 8 and 9 wherein said yeast expression vector further comprises a terminator attached to the 3' end of the inserted DNA coding segment.

- 20 11. A method according to any of claims 8 and 9 wherein said yeast expression vector further comprises a bacterial cell replication origin and is capable of replicating in a bacterial cell.

12. A method according to claim 10 wherein said  
25 terminator comprises the yeast alcohol dehydrogenase I terminator.

13. A method according to claim 10 wherein said terminator comprise the yeast GAPDH terminator.

14. A method according to claim 10 wherein said  
30 terminator comprises the yeast PyK terminator.

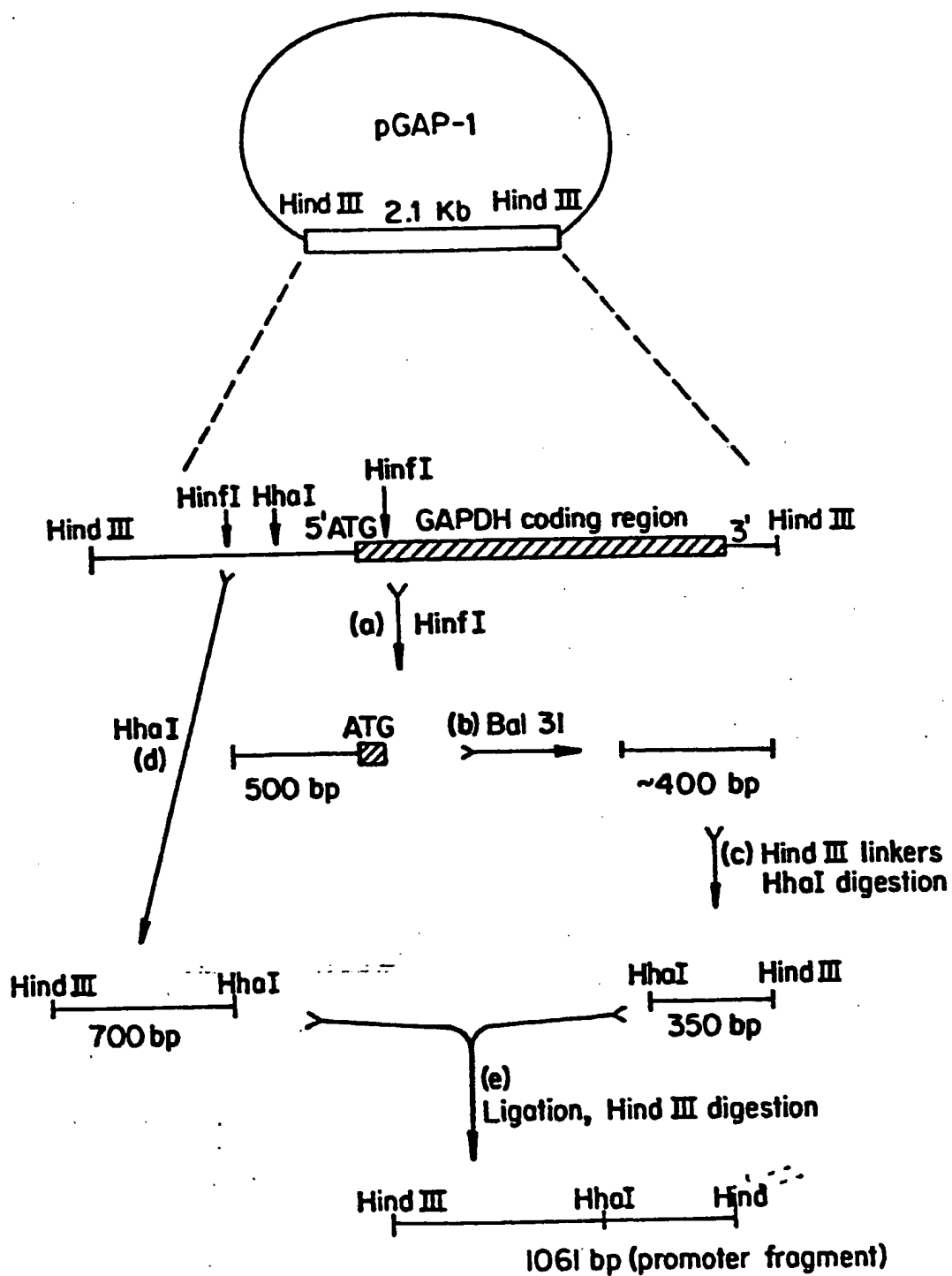


FIG. 1.

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0120551

DNA 347

10 20 30 40 50 60  
AAGCTTACCA GTTCTCACAC GGAACACCAC TAATGGACAC AAATTCGAAA TACTTTGACC

70 80 90 100 110 120  
CTATTTTCGA GGACCTTGTC ACCTTGAGCC CAAGAGAGCC AAGATTITAA TTTTCCTATG

130 140 150 160 170 180  
ACTTGAATGCA AATTCCCAAA GCTAATAACA TGCAAGACAC GTACGGSTCAA GAAGACATAT

190 200 210 220 230 240  
TTGACCTCTT AACTGGTTCA GACGCGACTG CCTCATCAGT AAGACCCGTT GAAAAGAAGT

250 260 270 280 290 300  
TACCTGAAAA AAACGAATAT ATACTAGCGT TGAATGTTAG CGTCAACAAC AAGAAGTTTA

310 320 330 340 350 360  
ATGACGCGGA GGCCAAGGCA AAAAGATTCC TTGATTACGT AAGGGAGTTA GAATCATTTT

370 380 390 400 410 420  
GAATAAAAAA CACGCTTTTT CAGTTCGAGT TTATCATTAT CAATACTGCC ATTTCAAAGA

430 440 450 460 470 480  
ATACGTAAAT AATTAATAGT AGTGATTTTC CTAACCTTAT TTAGTCAAAA ATTAGCCTTT

490 500 510 520 530 540  
TAATTCTGCT GTAACCCGTA CATGCCCAAA ATAGGGGGCG GGTACACAG AATATATAAC

550 560 570 580 590 600  
ATCGTAGGTG TCTGGGTGAA CAGTTTATCC CTGGCATCCA CTAAATATAA TGGAGCTCGC

610 620 630 640 650 660  
TTTTAAGCTG GCATCCAGAA AAAAAAGAA TCCCAGCACC AAAATATTGT TTTCTTCACC

670 680 690 700 710 720  
AACCATCAGT TCATAGGTCC ATTCTCTTAG CGCAACTACA GAGAACAGGG GCACAAACAG

730 740 750 760 770 780  
GCAAAAAACG GGCACAACCT CAATGGAGTG ATGCAACCTG CCTGGAGTAA ATGATGACAC

790 800 810 820 830 840  
AAGGCAATTG ACCCAGCAT GTATCTATCT CATTTTCTTA CACCTTCTAT TACCTTCTGC

850 860 870 880 890 900  
TCTCTCTGAT TTGAAAAAG CTGAAAAAA AGGTTGAAAC CAGTTCCCTG AAATTATTCC

910 920 930 940 950 960  
CCTACTTGAC TAATAAGTAT ATAAAGACGG TAGGTATTGA TTGTAATICT GTAAATCTAT

970 980 990 1000 1010 1020  
TTCTTAAGT TCTTAATTC TACTTTTATA GTTAGTCTTT TTTTITAGTT TAAACACCA

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FIG. 2

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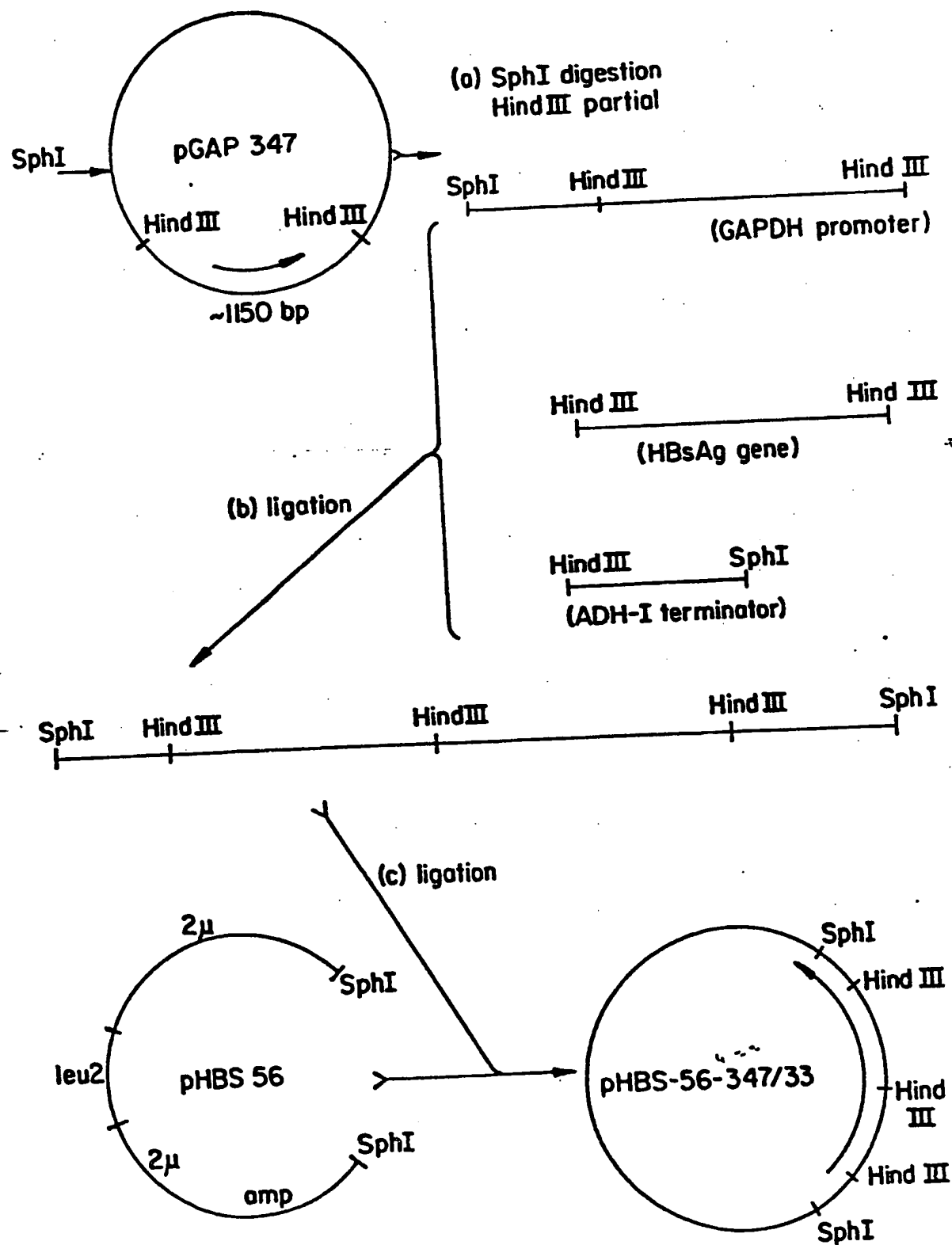


FIG 3

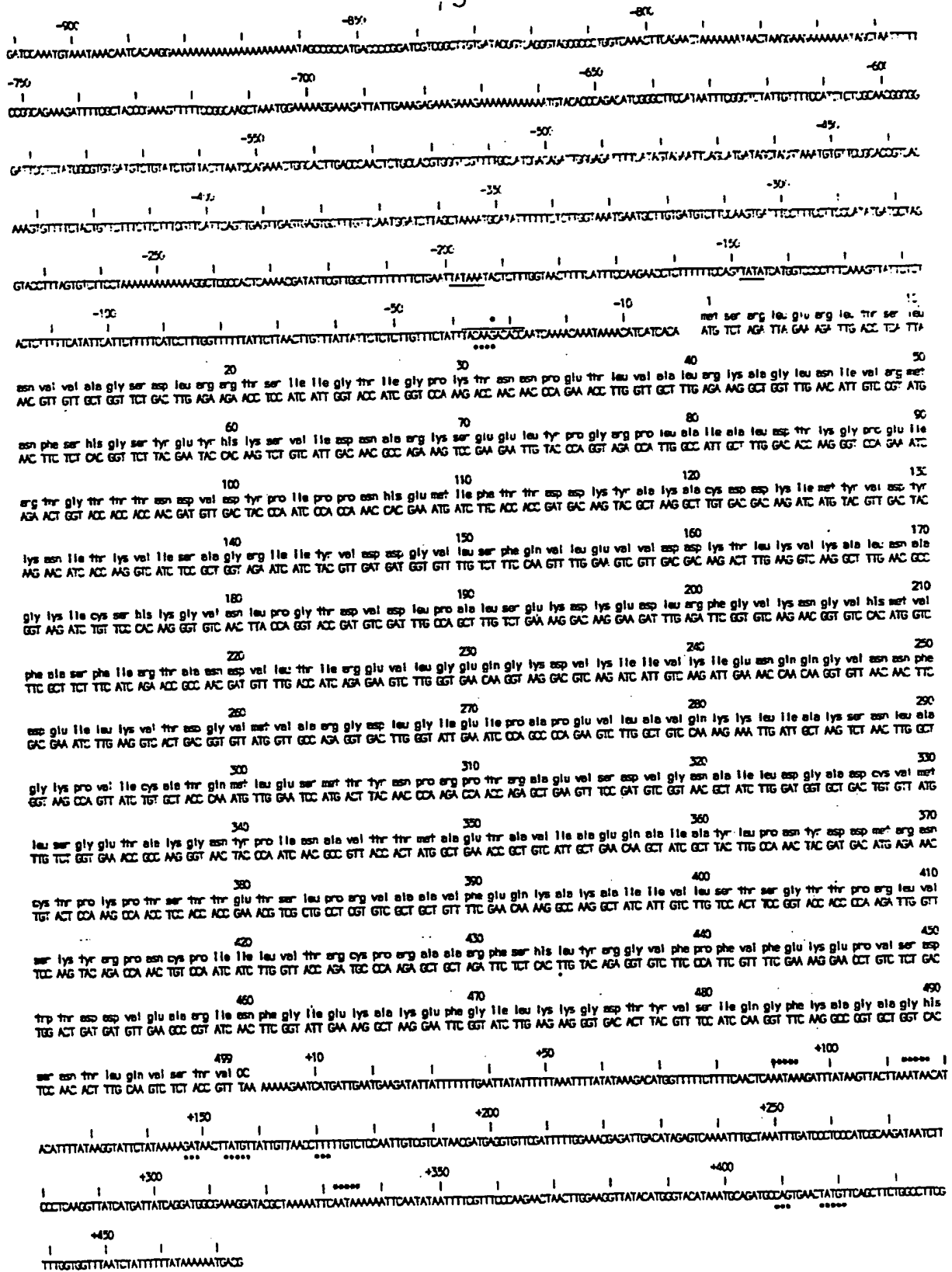


Fig. 4

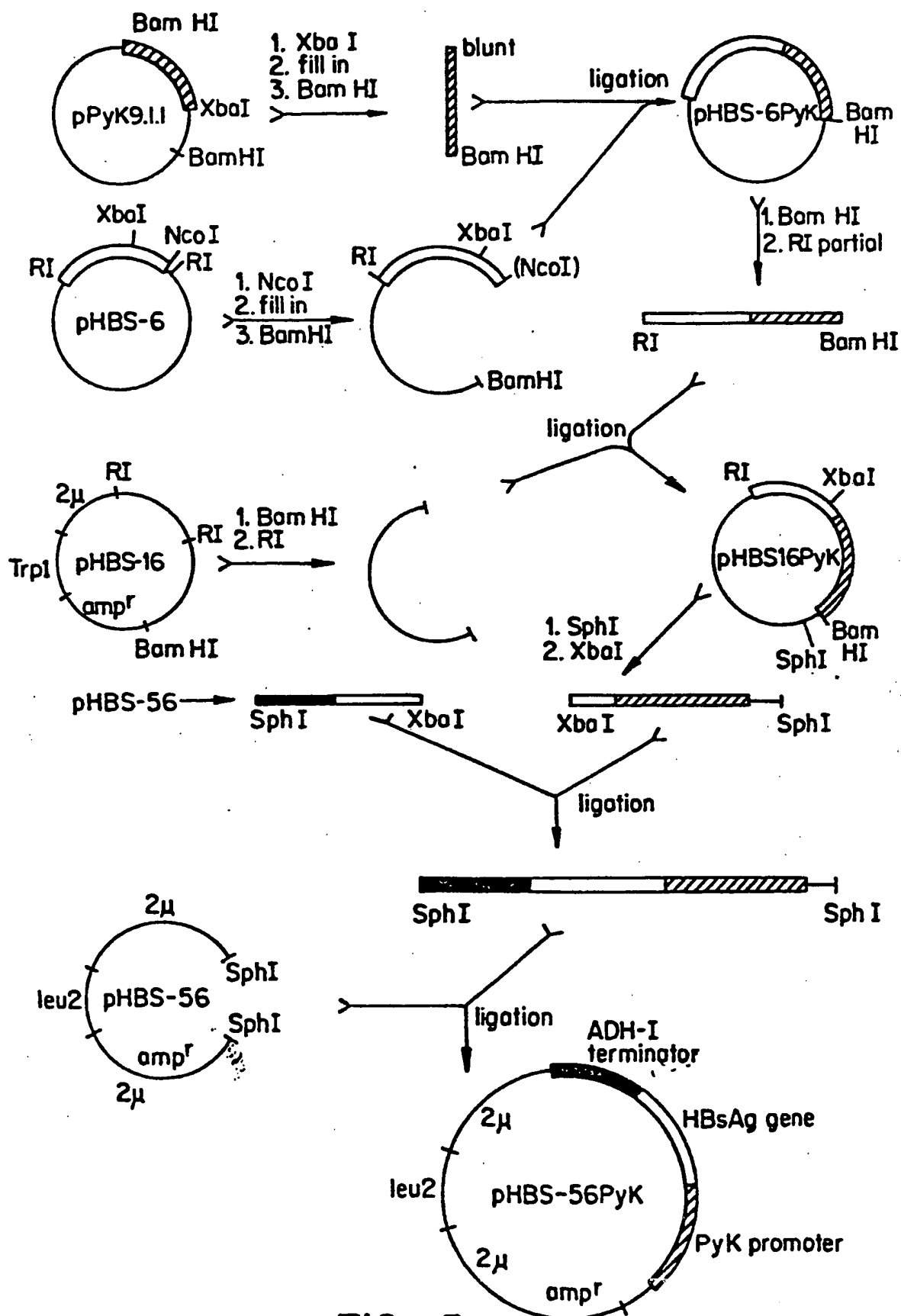


FIG. 5.

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(70) Proprietor: CHIRON CORPORATION  
4560 Horton Street  
Emeryville California 94608 (US)

(72) Inventor: Burke, Rae Lyn  
1624 8th Avenue  
San Francisco California (US)  
Inventor: Tekamp/Olson, Patricia  
1426 43rd Avenue  
San Francisco California (US)  
Inventor: Rosenberg, Steven  
3861 Fruitvale Avenue  
Oakland California (US)  
Inventor: Valenzuela, Pablo D. T.  
455 Upper Terrace No. 3  
San Francisco California (US)

(74) Representative: Glawe, Delfs, Moll & Partner  
Patentanwälte  
Postfach 26 01 62 Liebherrstrasse 20  
D-8000 München 26 (DE)

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## Description

## Background of the invention

For maximal expression of foreign genes in microbial systems it is usually advantageous to employ homologous regulatory elements within the expression vector. Efficiency of expression (product formation) is believed to be a function of and proportional to the strength of the promoter employed. In addition, regulation of gene expression by nutritional factors under the control of the experimenter offers further useful manipulatory tool. The glycolytic enzyme genes of yeast, e.g., those coding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate kinase (PyK), possess the above useful properties, i.e., high levels of expression (and thus by inference very efficient promoters) and susceptibility to regulation by components of the growth medium. For example, GAPDH can comprise as much as 5% of the dry weight of commercial baker's yeast (Krebs, E. G., *J. Biol. Chem.* (1953) 200:471). Furthermore, these enzymes are also highly inducible. For example, when yeast cultures are shifted from growth on acetate to glucose, the activity of GAPDH increased up to 200-fold in proportion to the concentration of the sugar in the medium (Maitra, P. K. and Lobo, Z., *J. Biol. Chem.* (1971) 246:475). These results suggest that the transcriptional machinery of these genes is highly regulated, perhaps by the participation of DNA sequences present in the 5' non-coding flanking region of the genes.

This invention relates to the isolation, structure and the successful use in yeast expression plasmids of DNA fragments corresponding to the 5' non-coding regions of the regulatable yeast genes GAPDH and PyK. These fragments which contain DNA sequences with strong transcription-promoting activity are called "promoters". They are ideal components of DNA vectors for commercial production of large quantities of protein coded by foreign genes under their transcriptional control.

In addition, this invention encompasses yeast expression plasmids further comprising an appropriate terminator to a form a "cassette" of promoter-foreign gene-terminator. The presence of the terminator increases expression of the foreign DNA.

An early attempt to express foreign DNA in yeast failed (Beggs, J. D. *et al.*, *Nature* (1980) 283:285). In this report, the hemoglobin DNA (inserted with its own promoter) was transcribed but the RNA was not spliced. A variety of explanations for this result are possible, e.g., an incorrect location for the initiation of transcription and/or the poor ability of yeast cells to carry out splicing of intervening sequences (introns).

Three GAPDH genes of yeast have been cloned (Holland, M. J. *et al.*, *Basic Life Science* (1981) 19:291, but their promoters have not been used for constructing expression systems in yeast by recombinant DNA methods. The PyK gene has also been cloned, but by genetic complementation only (no structural studies performed) (Kawasaki, G. and Fraenel, D. G., *Biochem. Biophys. Res. Comm.* (1982) 108:1107). Other yeast promoters, e.g., that of alcohol dehydrogenase I (Valenzuela, P. *et al.*, *Nature* (1982) 298:347 and Hitzeman, R. A. *et al.*, *Nature* (1981) 293:717) and phosphoglycerate kinase (Tuite, M. F. *et al.*, *EMBO J.* (1982) 1:60, and Hitzeman, R. A. *et al.*, *Science* (1983) 219:620) have been linked to foreign genes to produce yeast expression but no terminators were used. The present invention provides new promoters for yeast expression systems and combines the advantages of highly expressive promoters with the enhanced expression found with appropriately ligated terminators.

A published European Patent Application (072 318) disclosed the construction of a yeast expression vector which, upon induction, expressed hepatitis B virus surface antigen (HBsAg) S-protein under control of the yeast alcohol dehydrogenase I (ADHI) promoter.

## Brief description of the invention

This invention relates to a yeast expression vector comprising a segment of foreign DNA, e.g., the coding for hepatitis B virus (HBV) surface antigen (HBsAg), under transcriptional control of a yeast GAPDH promoter. Terminators may also be appropriately attached. The expression vector typically has a yeast replication origin and is capable of replicating in either type of cell. The expression vector, when used to transform yeast cells, will yield substantial amounts of the protein coded by the segment of foreign DNA.

## Brief description of the drawings

Figure 1: Isolation and tailoring of a GAPDH promoter fragment.

Figure 2: DNA sequence of the GAPDH promoter fragment.

Figure 3: Construction of a yeast expression plasmid containing the GAPDH promoter.

## Detailed description of the invention

In principle, yeast expression plasmids have particular advantages, including the following. Yeast can be grown in large-scale culture for commercial production by processes well-known in the art. In contrast, bacteria in large-scale culture are subject to the frequent problem of "phage-out". Yeast also appears to have much the same ability as mammalian cells to add carbohydrate groups to newly synthesized proteins, a capacity that bacteria do not have. Now that cDNA sequences are readily obtainable, the problem of expressing genes having introns is easily avoided.

The vectors of the present invention encompass promoters of unusually high efficiency. A promoter is defined herein as a DNA segment capable of functioning to initiate transcription of an adjoining DNA segment. Transcription is the synthesis of RNA (herein termed messenger RNA or mRNA), complementary

to one strand of the DNA adjoining the promoter region. In eukaryotes, messenger RNA synthesis is catalyzed by an enzyme termed RNA polymerase II. The minimum essential elements of promoter function are the following: To provide a starting point for the initiation of transcription and to provide a binding site for RNA polymerase II near the start site permitting selection of the proper strand of DNA as a template for messenger RNA synthesis. In addition, a eukaryotic promoter functions to regulate the relative efficiency of transcription of coding segments under its control. An active promoter is one which elicits synthesis of relatively large amounts of mRNA complementary to a strand of the adjacent DNA coding segment.

The structural correlates of promoter function have not been clearly established. A promoter segment usually can be identified in nature as a region lying adjacent to the 5' end of a given structural gene. (References to the 5' and 3' ends of a gene will be understood to indicate the corresponding respective ends of mRNA transcribed therefrom, and these, in turn, will be understood to correlate with the NH<sub>2</sub>— and —COOH termini of the encoded protein, respectively). Comparisons of the nucleotide sequences of promoters for various genes from various species have revealed only a few short regions of nucleotide sequence similarity in common among them. Most notable of these is the "TATA Box," a segment of about 5 to 10 nucleotides located generally about 70 to 230 nucleotides upstream from the site of transcription initiation, having a sequence generally resembling TATAA. For review of structural comparisons see Breathnach, R. and Chambon, P., *Ann. Rev. of Biochem.* (1981) 50:349. The TATA Box is believed to function in initiation of transcription.

The foreign gene will be free or substantially free of codons from the normal structural gene associated with the promoter. Usually, the foreign gene will be joined to a non-coding 3'-end of the regulatory region encompassing the promoter, so as to be free of the amino acids at the N-terminus of endogenous gene naturally associated with the regulatory region. That is, fewer than about 3 codons (9 nucleotides) will be retained with the regulatory region when joined to the foreign gene.

The presence of the terminator sequence at the 3' end of the coding segment enhances expression. The effect is generally similar to the addition of *rho* factor to prokaryotic transcription systems, wherein the rate of the release of RNA polymerase is enhanced to produce an increase in the rate of reinitiation of transcription. It will be understood that, while the terminator sequences are not required for detectable expression of foreign DNA segments, it is preferable to appropriately link them to enhance expression. The terminator region may be naturally associated with the same or different structural gene as the promoter region.

The most appropriate DNA vector for the GAPDH construction of this invention is a shuttle vector. These vectors can "shuttle" between a bacterial strain, such as *E. coli*, and yeast, since they have a bacterial origin of replication and a yeast origin of replication, see, e.g., Ammerer, G. *et al.*, *Recombinant DNA, Proc. Third Cleveland Symposium Macromolecules* (Walton, A. G., ed.), p. 185; Elsevier, Amsterdam (1981). A typical bacterial origin of replication is derived from, e.g., pBR322. The most useful yeast origin of replication is found in the extrachromosomal genetic element known as the 2 micron circle. In laboratory strains the 2 micron plasmid DNA is found in approximately 50 copies per cell and is stably maintained. For a review, see, for example, *Curr. Topics Micro. Imm.* (1982) 96:119. This yeast plasmid has also been sequenced (Hartley, J. L. *et al.*, *Nature* (1980) 286:860).

Representative samples of the plasmids and host cells used in the constructions of this invention have been placed on deposit with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, Plasmid pPyK 9.1.1 and yeast cell transformants 2150-2-3/pHBS-56 GAP347/33 and 2150-2-3/pHBS56PyK were placed on deposit on February 18, 1983 and have received ATCC Accession numbers 40061, 20665 and 20886, respectively.

In the Examples that follow, many of the techniques, reactions and separation procedures are already well-known in the art. All enzymes, unless otherwise stated, are available from one or more commercial sources, such as New England Biolabs, Beverly, Massachusetts; Collaborative Research, Waltham, Massachusetts; Miles Laboratories, Elkhart, Indiana; Boehringer Biochemicals, Inc., Indianapolis, Indiana and Bethesda Research Laboratories, Rockville, Maryland. Buffers and reaction conditions for restriction enzyme digestion were used according to recommendations supplied by the manufacturer for each enzyme, unless otherwise indicated. Standard methodology, for other enzyme reactions, gel electrophoresis separations and *E. coli* transformation may be found in *Methods in Enzymology*, (1979) 68. Transformation of yeast protoplasts can be carried out essentially as described by Beggs, *Nature* (1978) 275:104.

*E. coli* strains useful for transformation include X1776; K12 strain 294 (ATCC No. 31446); RR1 and HB101. Yeast strains XV610-8c having the genotype (*a ade2 ade6 leu2 lys1 trp1 can1*) and GM-3C-2, genotype: (*Leu2 Trp1 His4 CYC1-1CYP3-1*) (Faye, G. *et al.*, *Proc. Natl. Acad. Sci.* (1981) 78:12258) can be typically used for yeast transformations. It would be understood, however, that virtually any strain of yeast is useful for transformation. Bacteria can be grown and selected according to procedures described by Miller, J. H., *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1972). Yeast can be grown on the following media: YEPD containing 1% (w/v) yeast extract, 2% (w/v) peptone and (w/v) glucose; and, in the case of plating medium, 3% (w/v) agar. YNB plus CAA contains 6.7 grams of yeast nitrogen base (Difco Laboratories, Minneapolis, Minnesota), 10 mg of adenine, 10 mg of uracil, 5 g casamino acids (CAA) (Difco), 20 g glucose; and, in the case of plating media, 30 g agar per liter.

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Selection for tryptophan prototrophy can be made on plates containing 6.7 g yeast nitrogen base (lacking amino acids), supplemented for all growth requirements of the strain to be transformed except tryptophan.

### Example 1

#### 5 Cloning of the yeast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

A complementary DNA (cDNA) containing the yeast GAPDH coding sequences was prepared in the following manner:

PolyA<sup>+</sup> RNA was isolated from yeast strain A364A. Double-stranded cDNA was synthesized using AMV reverse transcriptase and *E. coli* DNA polymerase I. Poly-dC-tails were added to the double-stranded cDNA molecule using deoxynucleotide terminal transferase. Poly-dC-tailed cDNA was annealed to poly-dG-tailed pBR322 and used to transform *E. coli* HB101. One thousand transformants were screened by colony hybridization to labeled PolyA<sup>+</sup> RNA, and a subset further examined by restriction endonuclease mapping, and DNA sequencing. Three clones containing GAPDH sequences were isolated from the pool. One clone (pcGAP-9) contained an insert of about 1200 base pairs (bp) and was used for further work.

15 A yeast gene library was prepared by inserting fragments obtained after partial digestion of total yeast DNA with restriction endonuclease *Sau3A* into lambda phage Charon 28, according to Blattner, F. R. *et al.*, *Science* (1977) 196:161-169. Several fragments containing yeast GAPDH coding sequences were isolated by screening the phage library with labeled DNA from pcGAP-9. The yeast GAPDH gene of one of these clones was subcloned in pBR322 as a 2.1kb *HindIII* fragment (pGAP-1, see Figure 1) or as a 3.5kb *BamHI* fragment (pGAP-2). The GAPDH promoting-active fragments were isolated from these clones. The *HindIII*-*HhaI* fragment of about 800bp was ligated to the *HhaI*-*HindIII* fragment of about 350bp. The resulting 1061bp *HindIII* fragment was isolated by gel electrophoresis and cloned in pBR322, (pGAP-347), and the sequence determined (see Figure 2).

#### 25 Example 2

Construction of yeast vectors containing the GAPDH promoter, active in the expression of HBsAg.

A plasmid vector (pHBS-56GAP347/33), for the expression of HBV surface antigen in yeast, using the GAPDH promoter fragment was constructed as depicted in Figure 3.

Total digestion of pGAP-347 with *SphI* followed by partial digestion with *HindIII* yielded an approximately 1700bp *SphI*-*HindIII* fragment having about 1060bp of GAPDH promoter and about 530bp of pBR322. The 1700bp *SphI*-*HindIII* GAPDH promoter fragment was ligated with the 840bp *HindIII*-*HindIII* fragment (containing the HBsAg coding region, 26 bases of 5' non-coding region and 128bp of 3' non-coding region, obtained from pHBS-56) and then with the 350bp *HindIII*-*SphI* fragment containing the ADH-1 termination region (isolated from pHBS-56). The 2900bp *SphI* fragment (cassette) was isolated and cloned in pHBS-56 previously digested with *SphI*. The plasmid pHBS-56 (ATCC Accession No. 40047) has been described in a co-pending application (EPA No. 82.401473.2 published as no. 72318, of Regents of the University of California, herein incorporated by reference) and contains the entire 2 micron plasmid, in addition to a region with the yeast *leu2* gene and the amp resistance locus of pBR322. The resulting plasmid (pHBS-56GAP347/33) in which the promoter, gene and termination regions were in the proper orientations was isolated and used to transform yeast strain AB102 (*MATa, pep 4-3, leu 2-3 leu2-112, ura 3-52, his 4-580, cir<sup>r</sup>*) or strain 2150-2-3 (*MATa, ade1, leu2-04, cir<sup>r</sup>*). Strain AB102 is derived from SF657-9c by curing of 2 micron plasmids. Strain 2150-2-3 is from the collection of Dr. Leland Hartwell at the University of Washington.

#### 45 Example 3

Synthesis of HBsAg in yeast under GAPDH promoter control (plasmid pHBS-56GAP347/33).

One hundred ml cultures of strain AB102 containing plasmid pHBS56-347/33 were grown to optical density at 650nm of 1. Cell-free lysates were prepared by agitation with glass beads and removal of cell debris by centrifugation. HBsAg was measured by the Abbott Ausriall radioimmunoassay and protein concentration was determined by the Coomassie blue binding method. The results are shown in Table 1. They indicate that the GAPDH promoter is about 5 times more effective than the ADH-1 promoter for protein product expression in yeast.

TABLE 1  
Synthesis of HBsAg in yeast

(a) control from pHBS-56 (ADH-I promoter)			
Exp#	sAg (μg/ml)	Protein (mg/ml)	Spec. activity (μgsAg/mg protein)
1	8.8	18	0.49
2	14	25	0.56
3	12.4	20	0.62

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TABLE 2 (continued)  
(b) from pHBS-56GAP347/33 (GAPDH promoter)

Exp#	sAg ( $\mu$ g/ml)	Protein (mg/ml)	Spec. activity ( $\mu$ gAg/mg protein)
1	36	14	2.6
2	35	12	2.9
3	37	12.5	3.0

Similar results were obtained by substituting yeast strain 2150-2-3 for yeast strain AB102 and repeating Example 3.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

## Claims for the Contracting States: BE CH DE FR GB IT LI NL LU SE

1. A yeast expression vector comprising a segment of foreign DNA under transcriptional control of a yeast glyceraldehyde-3-phosphate dehydrogenase promoter having the sequence shown in Figure 2, said segment being in the correct orientation for transcription and having fewer than three codons from yeast glyceraldehyde-3-phosphate dehydrogenase at the 5'-end of said foreign DNA.
2. A yeast expression vector of claim 1 further comprising a terminator attached to the 3' end of the segment of foreign DNA.
3. A yeast expression vector according to claim 1, further comprising yeast two micron plasmid DNA or portion thereof.
4. A yeast expression vector of claim 1 wherein said foreign DNA codes for hepatitis B surface antigen or portion thereof.
5. The plasmid pHBS-56 bAP347/33 deposited in a yeast host under the ATCC accession number 20665.
6. A method of expressing a DNA coding segment in yeast, comprising the steps of:
  - (a) inserting the coding segment in a yeast expression vector, said vector comprising a DNA segment derived from a yeast glyceraldehyde-3-phosphate dehydrogenase promoter having the sequence shown in Figure 2 and having fewer than three codons from the 5'-end of glyceraldehyde-3-phosphate dehydrogenase, said promoter being adjacent to the 5' end of the inserted DNA coding segment and so oriented that transcription initiated within said promoter includes the coding segment, thereby providing a coding segment expression vector, and
  - (b) transforming yeast cells with the coding segment expression vector.
7. A method according to claim 6 wherein said yeast expression vector further comprises a terminator attached to the 3' end of the inserted DNA coding segment.
8. A method according to claim 6 wherein said yeast expression vector further comprises a bacterial cell replication origin and is capable of replicating in a bacterial cell.
9. A method according to claim 7 wherein said terminator comprises the yeast alcohol dehydrogenase terminator.
10. A method according to claim 7 wherein said terminator comprises the yeast glyceraldehyde-3-phosphate dehydrogenase (bAPDH) terminator.
11. A method according to claim 7 wherein said terminator comprises the yeast pyruvate kinase (PyK) terminator.

## Claims for the Contracting State: AT

1. A method of expressing a DNA coding segment in yeast, comprising the steps of:
  - (a) inserting the coding segment in a yeast expression vector, said vector comprising a DNA segment derived from a yeast glyceraldehyde-3-phosphate dehydrogenase promoter having the sequence shown in Figure 2 and having fewer than three codons from the 5'-end of glyceraldehyde-3-phosphate dehydrogenase, said promoter being adjacent to the 5' end of the inserted DNA coding segment and so oriented that transcription initiated within said promoter includes the coding segment, thereby providing a coding segment expression vector, and
  - (b) transforming yeast cells with the coding segment expression vector.

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2. A method according to claim 1 wherein said yeast expression vector further comprises a terminator attached to the 3' end of the inserted DNA coding segment.
3. A method according to claim 1 wherein said yeast expression vector further comprises a bacterial cell replication origin and is capable of replicating in a bacterial cell.
- 5 4. A method according to claim 2 wherein said terminator comprises the yeast alcohol dehydrogenase terminator.
5. A method according to claim 2 wherein said terminator comprises the yeast glyceraldehyde-3-phosphate dehydrogenase (bAPDH) terminator.
6. A method according to claim 2 wherein said terminator comprises the yeast pyruvate kinase (PyK) terminator.
- 10 7. A method according to claim 1 wherein said yeast expression vector further comprises the yeast two micron plasmid DNA or portion thereof.
8. A method according to claim 1 wherein said foreign DNA codes for hepatitis B surface antigen or portion thereof.
- 15 9. A method according to claim 1 wherein said coding expression vector is the plasmid pHBS-56 bAP347/33 deposited in a yeast host under the ATCC accession number 20665.

### Patentansprüche für die Vertragsstaaten: BE CH DE FR GB IT LI NL LU SE

- 20 1. Hefeexpressionsvektor mit einem Abschnitt einer Fremd-DNA unter Transkriptionskontrolle eines Hefe-Glyceraldehyd-3-phosphat-dehydrogenase-Promotors mit einer in Fig. 2 wiedergegebenen Sequenz, wobei sich der Abschnitt in der korrekten Orientierung für die Transkription befindet und weniger als 3 Codons von der Hefe-Glyceraldehyd-3-phosphat-dehydrogenase an dem 5'-Ende der Fremd-DNA aufweist.
- 25 2. Hefeexpressionsvektor nach Anspruch 1, der weiterhin einen am 3'-Ende des Abschnittes der Fremd-DNA angeordneten Terminator aufweist.
3. Hefeexpressionsvektor nach Anspruch 1, der weiterhin eine Hefe-2-micron-Plasmid-DNA oder einen Teil derselben aufweist.
4. Hefeexpressionsvektor nach Anspruch 1, bei dem die Fremd-DNA für ein Hepatitis B-Oberflächenantigen oder einen Teil desselben kodiert.
- 30 5. Plasmid pHBS-56GAP347/33, hinterlegt in einem Hefe-Wirtsstamm unter der ATCC-Zugangsnummer 20665.
6. Verfahren zur Expression eines DNA-Kodierungsabschnittes, umfassend die Schritte
- a) Einfügung des Kodierungsabschnittes in einen Hefeexpressionsvektor, der einen von einem Hefe-Glyceraldehyd-3-phosphat-dehydrogenase-Promotor mit einer in Fig. 2 wiedergegebenen Sequenz abgeleiteten DNA-Abschnitt umfaßt und weniger als 3 Codons von dem 5'-Ende der Hefe-Glyceraldehyd-3-phosphat-dehydrogenase aufweist, wobei der Promotor sich neben dem 5'-Ende des eingefügten DNA-Kodierungsabschnittes befindet und so orientiert ist, daß in dem Promotor eingeleitete Transkription den Kodierungsabschnitt einschließt und dadurch ein Kodierungsabschnitt-Expressionsvektor gebildet wird,
- 40 und
- b) Transformation der Hefezellen mit dem Kodierungsabschnitt-Expressionsvektor.
7. Verfahren nach Anspruch 6, bei dem der Hefeexpressionsvektor weiterhin einen am 3'-Ende des eingefügten DNA-Kodierungsabschnittes angeordneten Terminator aufweist.
8. Verfahren nach Anspruch 6, bei dem der Hefeexpressionsvektor weiterhin einen Bakterienzellen-Replikationsorigin aufweist und zur Replikation in einer Bakterienzelle befähigt ist.
- 45 9. Verfahren nach Anspruch 7, bei dem der Terminator den Hefe-Alkohol-dehydrogenase-Terminator umfaßt.
10. Verfahren nach Anspruch 7, bei dem der Terminator den Hefe-Glyceraldehyd-3-phosphat-dehydrogenase (GAPDH)-Terminator umfaßt.
- 50 11. Verfahren nach Anspruch 7, bei dem der Terminator den Hefe-Pyruvatkinase (PyK)-Terminator umfaßt.

### Patentansprüche für den Vertragsstaat: AT

- 55 1. Verfahren zur Expression eines DNA-Kodierungssegmentes in Hefe, umfassend die Schritte:
- a) Einfügung des Kodierungssegmentes in einen Hefeexpressionsvektor, wobei der Vektor einen von einem Hefe-Glyceraldehyd-3-phosphat-dehydrogenase-Promotor mit einer in Fig. 2 wiedergegebenen Sequenz abgeleiteten DNA-Abschnitt umfaßt und weniger als 3 Codons von dem 5'-Ende der Hefe-Glyceraldehyd-3-phosphat-dehydrogenase aufweist, wobei der Promotor sich neben dem 5'-Ende des eingefügten DNA-Kodierungsabschnittes befindet und so orientiert ist, daß die in dem Promotor eingeleitete Transkription den Kodierungsabschnitt einschließt und dadurch ein Kodierungsabschnitt-Expressionsvektor gebildet wird, und
- 60 b) Transformation der Hefezellen mit dem Kodierungsabschnitt-Expressionsvektor.
2. Verfahren nach Anspruch 1, bei dem der Hefeexpressionsvektor weiterhin einen Terminator umfaßt, der am 3'-Ende des eingefügten DNA-Segmentes angeordnet ist.
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3. Verfahren nach Anspruch 1, bei dem der Hefeexpressionsvektor weiterhin einen Bakterienzellen-Replikationsorigin umfaßt und in einer Bakterienzelle replizierbar ist.
4. Verfahren nach Anspruch 2, bei dem der Terminator den Hefe-Alkohol-dehydrogenase-Terminator umfaßt.
- 5 5. Verfahren nach Anspruch 2, bei dem der Terminator den Hefe-Glyceraldehyd-3-phosphat-dehydrogenase(GAPDH)-Terminator umfaßt.
6. Verfahren nach Anspruch 2, bei dem der Terminator den Hefe-Pyruvasekinase(PyK)-Terminator umfaßt.
7. Verfahren nach Anspruch 1, bei dem der Hefeexpressionsvektor weiterhin eine Hefe-2-micron-Plasmid-DNA oder Teile derselben umfaßt.
- 10 8. Verfahren nach Anspruch 1, bei dem die Fremd-DNA für Hepatitis B-Oberflächenantigen oder Teile desselben kodiert.
9. Verfahren nach Anspruch 1, bei dem der Kodierungs-Expressionsvektor das Plasmid pHBS-56GAP347/33, hinterlegt in einem Hefe-Wirtstamm mit der Zugangsnummer ATCC 20665, ist.

15

### Revendications pour les Etats contractants: BE CH DE FR GB IT LI NL LU SE

1. Vecteur d'expression pour levure, comprenant un segment d'ADN étranger sous régulation de transcription d'un promoteur de glyceraldéhyde-3-phosphate-déshydrogénase de levure ayant la séquence représentée sur la fig. 2, ce segment étant dans l'orientation correcte pour la transcription et ayant moins de trois codons de glyceraldéhyde-3-phosphate-déshydrogénase de levure à l'extrémité 5' dudit ADN étranger.
2. Vecteur d'expression pour levure selon la revendication 1, comprenant en outre un terminateur attaché à l'extrémité 3' du segment d'ADN étranger.
3. Vecteur d'expression pour levure selon la revendication 1, comprenant en outre un ADN à plasmides de 2 microns de levure ou une partie de celui-ci.
4. Vecteur d'expression pour levure selon la revendication 1, dans lequel l'ADN étranger code pour l'antigène de surface de l'hépatite B ou pour une partie de celui-ci.
- 30 5. Plasmide pHBS-56 GAP 347/33 déposé dans une levure-hôte sous le numéro d'admission ATCC 20665.
6. Procédé d'expression d'un segment codant d'ADN dans une levure, comprenant les étapes consistant:
- (a) à insérer le segment codant dans un vecteur d'expression de levure, ce vecteur comprenant un segment d'ADN dérivé d'un promoteur de glyceraldéhyde-3-phosphate-déshydrogénase de levure ayant la séquence représentée sur la fig. 2 et ayant moins de trois codons de l'extrémité 5' de la glyceraldéhyde-3-phosphate-déshydrogénase, ledit promoteur étant adjacent à l'extrémité 5' du segment codant d'ADN inséré et étant orienté de telle sorte que la transcription initiée dans ledit promoteur comprenne le segment codant, ce qui donne un vecteur d'expression de segment codant, et
- 40 (b) à transformer des cellules de levure avec le vecteur d'expression de segment codant.
7. Procédé selon la revendication 6, dans lequel ledit vecteur d'expression pour levure comprend en outre un terminateur attaché à l'extrémité 3' du segment codant d'ADN inséré.
8. Procédé selon la revendication 6, dans lequel ledit vecteur d'expression pour levure comprend en outre une origine de la répllication de cellules bactériennes et est capable de répllication dans une cellule bactérienne.
- 45 9. Procédé selon la revendication 7, dans lequel ledit terminateur comprend le terminateur d'alcool-déshydrogénase de levure.
10. Procédé selon la revendication 7, dans lequel ledit terminateur comprend le terminateur de glyceraldéhyde-3-phosphate-déshydrogénase (GAPDH) de levure.
- 50 11. Procédé selon la revendication 7, dans lequel ledit terminateur comprend le terminateur de pyruvatekinase (PyK) de levure.

### Revendications pour l'Etat contractant: AT

- 55 1. Procédé d'expression d'un segment codant d'ADN dans une levure, comprenant les étapes consistant:
- (a) à insérer le segment codant dans un vecteur d'expression pour levure, ce vecteur comprenant un segment d'ADN dérivé d'un promoteur de glyceraldéhyde-3-phosphate-déshydrogénase de levure ayant la séquence représentée sur la fig. 2 et ayant moins de trois codons de l'extrémité 5' de la glyceraldéhyde-3-phosphate-déshydrogénase, ledit promoteur étant adjacent à l'extrémité 5' du segment codant d'ADN inséré et étant orienté de telle sorte que la transcription initiée dans ledit promoteur comprenne le segment codant, ce qui donne un vecteur d'expression de segment codant, et
- 60 (b) à transformer des cellules de levure avec le vecteur d'expression de segment codant.
2. Procédé selon la revendication 1, dans lequel ledit vecteur d'expression pour levure comprend en outre un terminateur attaché à l'extrémité 3' du segment codant d'ADN inséré.
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3. Procédé selon la revendication 1, dans lequel ledit vecteur d'expression pour levure comprend en outre une origine de la répllication de cellules bactériennes et est capable de répllication dans une cellule bactérienne.

5 4. Procédé selon la revendication 2, dans lequel ledit terminateur comprend le terminateur d'alcool-déshydrogénase de levure.

5. Procédé selon la revendication 2, dans lequel ledit terminateur comprend le terminateur de glyceraldéhyde-3-phosphat-déshydrogénase (GAPDH) de levure.

6. Procédé selon la revendication 2, dans lequel ledit terminateur comprend le terminateur de pyruvatekinase (PyK) de levure.

10 7. Procédé selon la revendication 1, dans lequel ledit vecteur d'expression pour levure comprend en outre un ADN à plasmides de 2 microns de levure ou une partie de celui-ci.

8. Procédé selon la revendication 1, dans lequel ledit ADN étranger code pour l'antigène de surface de l'hépatite B ou pour une partie de celui-ci.

15 9. Procédé selon la revendication 1, dans lequel ledit vecteur d'expression codant est le plasmide pHBS-56 GAP 347/33 déposé dans une levure-hôte sous le numéro d'admission ATCC 20665.

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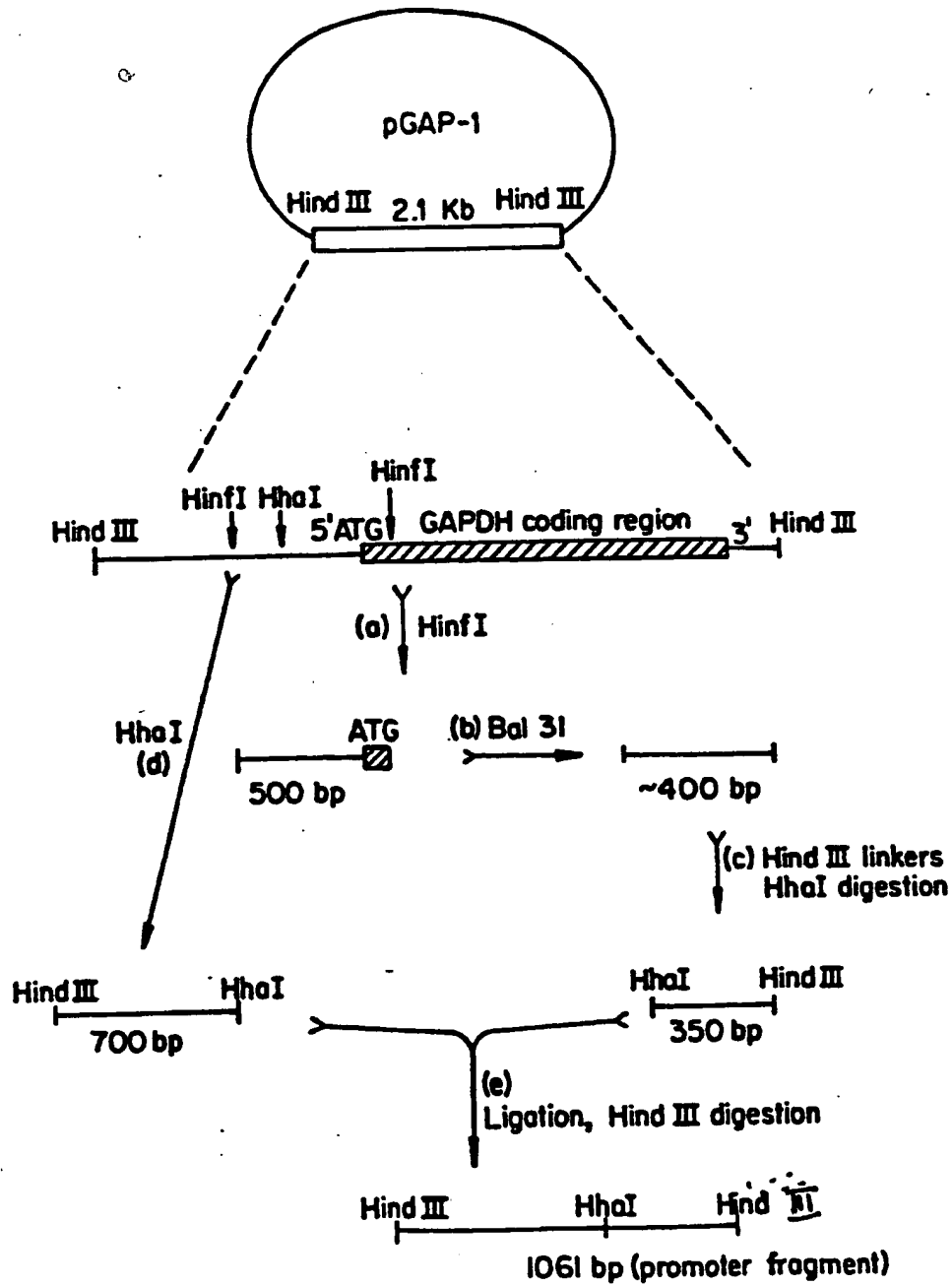


FIG. 2.



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DNA 347

10	20	30	40	50	60
AAGCTTACCA	GTTCACAC	GGAACACCAC	TAATGGACAC	AAATTCGAAA	TACTTTGACC
70	80	90	100	110	120
CTATTTTCGA	GGACCTTGTC	ACCTTGAGCC	CAAGAGAGCC	AAGATTIAAA	TTTTCCTATG
130	140	150	160	170	180
ACTTGATGCA	AATTCCTAAA	GCTAATAACA	TGCAAGACAC	GTACGGTCAA	GAAGACATAT
190	200	210	220	230	240
TTGACCTCTT	AACGGTTTCA	GACGCGACTG	CCTCATCAGT	AAGACCCGTT	GAAAAAGAACT
250	260	270	280	290	300
TACCTGAAAA	AAACGAATAT	ATACTAGCGT	TGAATGTTAG	CGTCAACAAC	AAGAAGTTTA
310	320	330	340	350	360
ATGACGCGGA	GGCCAAGGCA	AAAAGATTCC	TTGATTACGT	AAGGGAGTTA	GAATCATTTT
370	380	390	400	410	420
GAATAAAAAA	CACGCTTTTT	CAGTTCGAGT	TTATCATTAT	CAATACTGCC	ATTTCAAAGA
430	440	450	460	470	480
ATACGTAAT	AATTAATAGT	AGTGATTTTC	CTAACTTTAT	TTAGTCAAAA	ATTAGCCTTT
490	500	510	520	530	540
TAATTCGTCT	GTAACCCGTA	CATGCCCAAA	ATAGGGGGCG	GGTTACACAG	AATATATAAC
550	560	570	580	590	600
ATCGTAGGTG	TCTGGGTGAA	CAGTTTATCC	CTGGCATCCA	CTAAATATAA	TGGAGCTCGC
610	620	630	640	650	660
TTTTAAGCTG	GCATCCAGAA	AAAAAAGAA	TCCCAGCACC	AAAATATTGT	TTTCTTCACC
670	680	690	700	710	720
AACCATCAGT	TCATAGGTCC	ATTCTCTTAG	CGCAACTACA	GAGAACAGGG	GCACAAACAG
730	740	750	760	770	780
GCAAAAAACG	GGCACAACCT	CAATGGAGTG	ATGCAACCTG	CCTGGAGTAA	ATGATGACAC
790	800	810	820	830	840
AAGGCAATTG	ACCCACGCAT	GTATCTATCT	CATTTTCTTA	CACCTTCTAT	TACCTTCTGC
850	860	870	880	890	900
TCTCTCTGAT	TTGAAAAAAG	CTGAAAAAAA	AGGTTGAAAC	CAGTTCCCTG	AAATTATTCC
910	920	930	940	950	960
CCTACTTGAC	TAATAAGTAT	ATAAAGACGG	TAGGTATTGA	TTGTAATTCT	GTAAATCTAT
970	980	990	1000	1010	1020
TTCTTAAGCT	TCITAAATTC	TACTTTTATA	GTTAGTCTTT	TTTTTAGTTT	TAAACACCA
1030	1040	1050	1060		
AGAACTTAGT	TTCGAATAAA	CACACATAAA	CAAACAAGCT	T	

FIG. 2

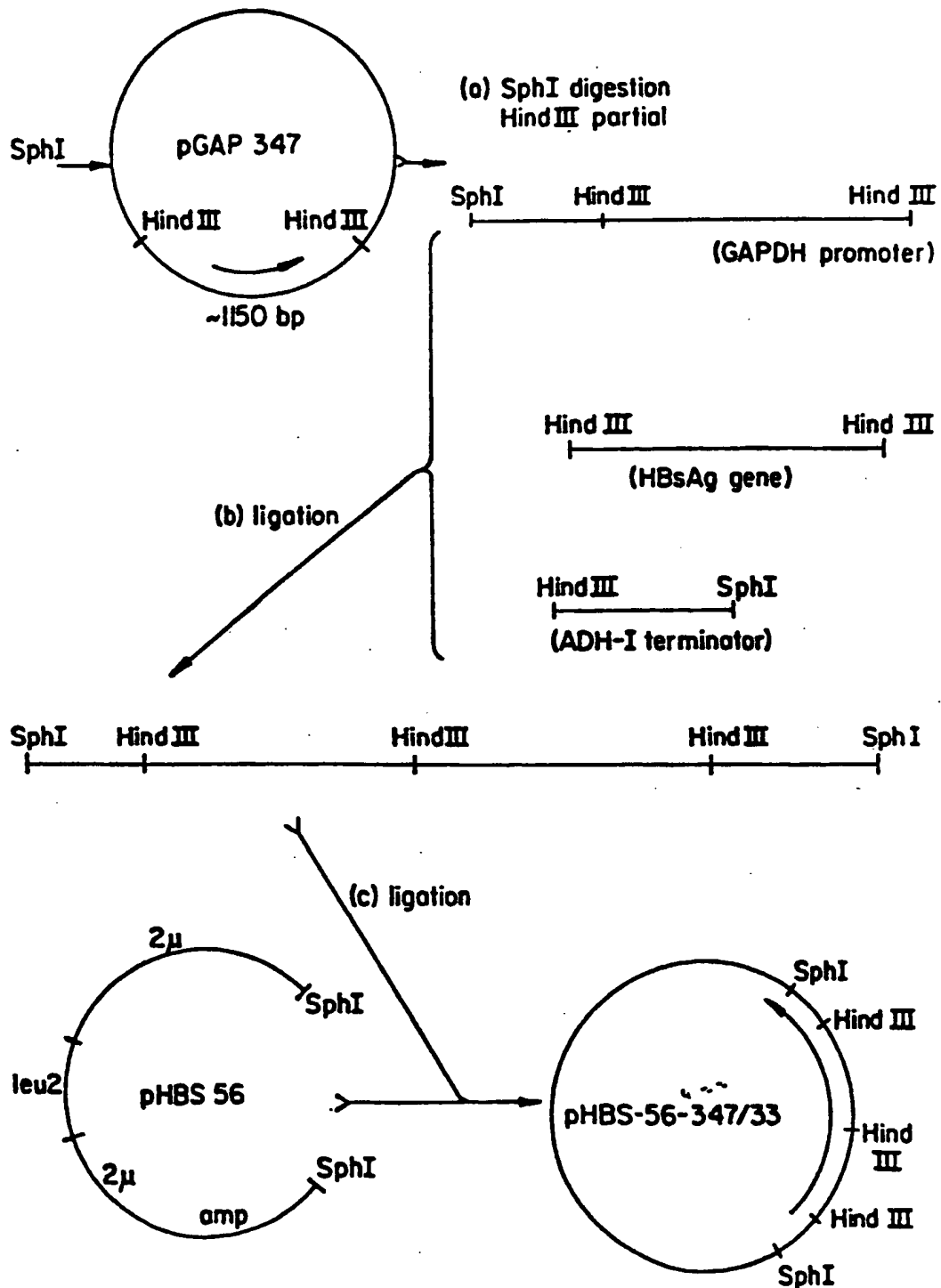


FIG. 3.

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004137598

WPI Acc No: 1984-283138/198446

XRAM Acc No: C84-120138

XRPX Acc No: N84-211294

Recovery of viruses and virus antigens - by liberation from fibronectin  
using an enzyme or detergent or by displacement or heat treatment

Patent Assignee: SEELIG R (SEEL-I)

Inventor: POTT G; SEELIG H

Number of Countries: 021 Number of Patents: 012

Patent Family:

Patent No	Kind	Date	Applicat No	Kind	Date	Week
DE 3316464	A	19841108	DE 3316464	A	19830505	198446 B
EP 124896	A	19841114	EP 84105066	A	19840504	198446
WO 8404326	A	19841108	WO 84EP134	A	19840504	198446
AU 8428650	A	19841119				198506
NO 8500025	A	19850311				198517
BR 8406854	A	19850319				198518
HU 35279	T	19850628				198533
JP 60501241	W	19850808	JP 84501830	A	19840504	198538
FI 8500051	A	19850104				198542
DK 8500056	A	19850305				198546
EP 124896	B	19900131				199005
DE 3481199	G	19900308				199011

Priority Applications (No Type Date): DE 3316464 A 19830505

Patent Details:

Patent No	Kind	Lan	Pg	Main IPC	Filing Notes
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DE 3316464	A		23		
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EP 124896	A	G			
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Designated States (Regional): AT BE CH DE FR GB IT LI LU NL SE

WO 8404326	A	G			
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Designated States (National): AU BR DK FI JP MC NO SU US

EP 124896	B	G			
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Designated States (Regional): AT BE CH DE FR GB IT LI LU NL SE

Abstract (Basic): EP 124896 A

Process for obtaining viruses or viral antigen from biological material, especially body fluids, characterized in that the starting material used is a fibronectin-containing body fluid or a biological material to which fibronectin has been added, and the virus or viral antigen is released by destruction, which is known per se, of the

mechanism of binding, especially by specific enzymes and/or detergents or by displacement reactions or heating, and, where appropriate, the viruses

or the viral antigen is isolated from the resulting suspension and, if desired, further purified in a manner known per se.

(8pp)

DE 3316464 A

Recovery of viruses or virus antigens from biological material esp. body fluids comprises using a fibronectin-contg. body fluid or a biological material mixed with fibronectin as the starting material and liberating the virus or virus antigen by destroying the binding mechanism esp. using a specific enzyme and/or detergent or by a displacement reaction or by heating and then isolating the virus or

virus antigen from the suspension obtd. and opt. purifying.

Pref. fibronectin fragments capable of bonding with the virus or antigen are used instead of fibronectin. Pref. the body fluid is blood and the biological material is faeces homogenisate. Pref. the enzyme is Neuraminidase or chymotrypsin.

USE/ADVANTAGE - The virus and virus antigens can be used for diagnostic purposes, e.g. in the diagnosis of Non-A Non-B-hepatitis. The virus and virus antigen can also be used in the prepn. of vaccine.

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Abstract (Equivalent): EP 124896 B

Process for obtaining viruses or viral antigen from biological material, especially body fluids, characterized in that the starting material used is a fibronectin-containing body fluid or a biological material to which fibronectin has been added, and the virus or viral antigen is released by destruction, which is known per se, of the mechanism of binding, especially by specific enzymes and/or detergents or by displacement reactions or heating, and, where appropriate, the viruses or the viral antigen is isolated from the resulting suspension and, if desired, further purified in a manner known per se.